Optimizing Halogenation Conditions of *N*-Halamine Polymers and Investigating Mode of Bactericidal Action

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ABSTRACT: An *N*-Halamine biocidal polymer was prepared by co-polymerizing toluene-2,6-diisocyanate with a new heterocyclic uramil-based azo-monomer, followed by halogenation. The mode of action of *N*-halamine polymers on bacteria was investigated and halogenation conditions (temperature, halogenation time, halogen concentration) were optimized for bactericidal action against *E. coli* and *S. aureus*. It was found that the mode of action of this type of polymer is a combination of different factors; contact, release, and through interaction of the polymer with the bacterial medium. The most effective halogenation conditions were stirring 1 g polymer in 10 mL sodium hypochlorite (10 %) for 1 h at ambient temperature (22°C). © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 113: 2404–2412, 2009

Key words: azo polymers; copolymerization; halogenated; polyurethanes; antibacterial

INTRODUCTION

N-halamine polymers are an important type of biocidal polymers; usually prepared by incorporating heterocyclic rings on the polymer followed by halogenation.[1–22] The halogen attached to the heterocyclic rings, responsible for the biological activity, can be stabilized by introducing electron donating groups on the ring.[1–22] Different types of *N*-halamine polymers have been prepared, most of them carrying at most two or three halogen atoms per heterocyclic ring.[3–22] Recently, we described the preparation of *N*-halamine polymers incorporating up to 7 halogen atoms per repeating unit and their application as biocidal polymers.[1,2] The mode of action of *N*-halamine polymers is still unclear; opinion is divided between those who have explained the mode of action of these polymers as delivery of the halogen to the bacterial cells by direct contact only[10–12,22] and those who explain the mode of action as a release of halogen to the medium.[5] Herein we have focused on investigating these aspects of the mode of action and, in addition, the halogenation conditions have been varied to improve the biocidal action. To achieve this, three parameters were explored; temperature, time, and halogen concentration during the halogenation process. The biological activity of the prepared samples was examined against examples of Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) bacteria.

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EXPERIMENTAL

Materials

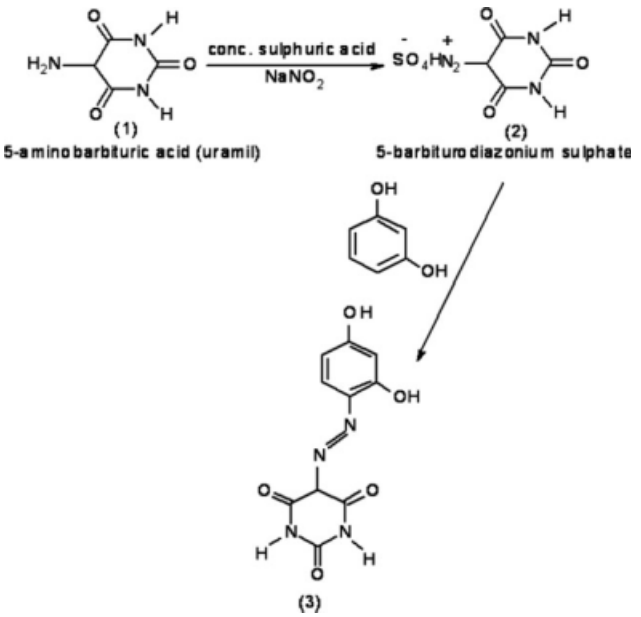
Barbituric acid, granulated tin, resorcinol, fuming nitric acid, sodium nitrite, tolylene-2,6-diisocyanate, sodium hypochlorite, bromine, and iodine were supplied by Sigma Aldrich Chemicals, UK. Sodium hydroxide, hydrochloric acid, sulphuric acid, sodium thiosulfate, starch, acetic acid, potassium iodide, and dimethylformamide were supplied by Fisher Chemicals, UK. Nutrient broth and Nutrient agar (Oxoid).

Polymer preparation

The polymers under investigation were prepared according to the methodology reported earlier,[1,2] as follows:

Diazotization of uramil

Uramil **(1)** (5-aminobarbituric acid) (1.40 g, 0.01 mol) was dissolved in 5 mL concentrated sulphuric acid. The temperature was kept at 0°C using an external ice bath. A cold solution of $NaNO_2$ [0.69 g of $NaNO_2$ (0.01 mol) + 10 mL water] was added dropwise to the uramil solution with stirring to form the uramil diazonium salt **(2)**, Scheme (1).[1,2]



Scheme 1 Diazotization of uramil and its coupling with resorcinol.

Preparation of 1,3-dihydroxy-4(5-azobarbituric acid)-benzene (3)

Resorcinol (1.1 g, 0.01 mol) and NaOH (5.5 g, 0.14 mol) were dissolved in 20 mL water and added gradually to cold uramildiazonium salt **(2)**. A dark purple product precipitated and this was filtered, washed copiously with cold water, dried and weighed, which produced 2.6 g (99% yield, melting point $> 360°C$), Scheme (2).[1,2]

Analysis, FTIR (KBr): 1603, 1705, 1411, 3100, 3432, and 2942 cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ 1.3 (s, 1H), 5.4 (s, 1H), 6.2 (s, 2H), 6.9–7.2 (s, 3H) and 10.2 (s, 1H). ^{13}C-NMR (DMSO-d_6): ppm 49, 102.4, 103, 105, 106, 129, 150.3, and 158.3. Elemental analysis, found (%): C, 45.1; H, 2.9; N, 20.9. Calculated for $C_{10}H_8N_4O_5$ (%): C, 45.5; H, 3; N, 21.2.[1,2]

Preparation of poly[(1,3-dihydroxy-4(5-azobarbituric acid)-benzene)-*co*– (tolylene-2,6-diisocyanate)] (polyurethane) (4)

Monomer **(3)** (2.6 g, 0.01 mol) and tolylene-2,6-diisocyanate (1.7 g, 0.01 mol) were heated in 30 mL dimethylformamide for 5 h at 90°C. The reaction was cooled and 50 mL of methanol was added. A brown product precipitated which was filtered, washed copiously with methanol, dried, and weighed. This produced 3.8 g (86% yield), Scheme (2).[1,2]

Analysis, FTIR (KBr): 1640, 1700, 1660, 3429, 1135, 1471, and 2920 cm^{-1}. ^{1}H-NMR (DMSO-d_6): δ 2.2 (s, 3H), 4.8 (s, 1H), 4.2 (s, 1H), 6.8 (s, 2H), 10.5 (s, 1H), and 7.0–8.4 (s, 6H). ^{13}C-NMR (DMSO-d_6): 11.4, 49, 109.9, 111.6, 113.2, 116,117.9, 118.5, 120, 121, 125, 137.3, 137.7, 146, 150, 185, 163.0, and 153.2 ppm. Elemental analysis, found (%): C, 51.7; H, 3.2; N, 18.1. Calculated for $C_{19}H_{16}N_8O_7$ (%): C, 52.1; H, 3.2; N, 19.2.[1,2]

There are no available data regarding the molecular weight of the polymer. The polymer is not soluble in organic solvents and only partially dissolves in dimethyl sulfoxide.

Halogenation of polyurethane (4) under different conditions

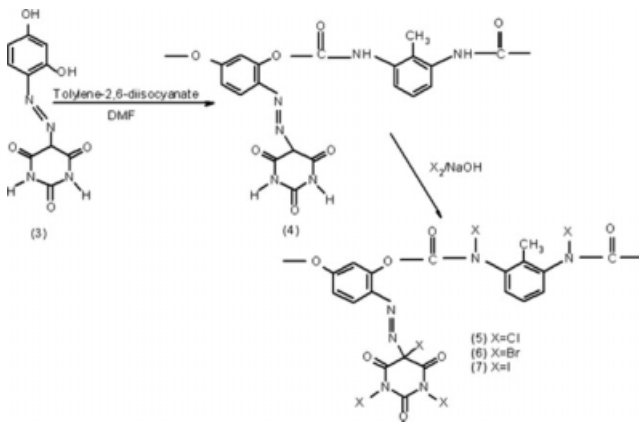
Halogenation conditions were changed methodically in order to optimize the process; in addition halogenation was carried out using sodium hypohalogenate instead of the more usual halogen gas.

Three parameters were tested: reaction time, temperature, and halogen concentration. The method in each case was to stir 1 g of the non-halogenated polymer **(4)** with sodium hypohalogenate under different conditions, Table I. The product from each experiment was filtered, washed copiously with water, and dried under vacuum.

Sodium hypohalogenate was prepared by adding the halogen to a solution of 10% sodium hydroxide in an ice bath until it attained pH 7. The quantity of halogen loaded onto each polymer was determined by iodometric titration,[10,22] Table II.

Biological activity of the *N*-halamine polymers: Varying parameters during the halogenation process

Bacterial suspensions of *S. aureus* (Gram-positive) and *E. coli* (Gram-negative) were prepared as described previously[2] and the biological activity of the polymers tested as follows: 0.25 g of each polymer sample was added to 10 mL of bacterial



Scheme 2 Preparation of poly[(1,3-dihydroxy-4(5-azobarbituric acid)-benzene)-*co*-(tolylene-2,6-diisocyanate)] (polyurethane) and its halogenation.

TABLE I
Different Halogenation Conditions during Sample Preparation

Polymer **(4)**	Experiment no.	Stirring time (h)	NaOX conc.	temperature
Optimizing halogenation time				
1 g	1	0.5	5%	Ambient temperature
1 g	2	1	5%	Ambient temperature
1 g	3	2	5%	Ambient temperature
1 g	4	4	5%	Ambient temperature
1 g	5	6	5%	Ambient temperature
Optimizing halogenation temperature				
1 g	1	1	5%	$< 5°C$
1 g	2	1	5%	Ambient temperature
1 g	3	1	5%	35°C
1 g	4	1	5%	45°C
Optimizing halogen concentration				
1 g	1	1	2.5%	Ambient temperature
1 g	2	1	5%	Ambient temperature
1 g	3	1	7.5%	Ambient temperature
1 g	4	1	10%	Ambient temperature

where X = Cl, Br, or I. Ambient temperature $\sim$ 22°C

suspension (either Gram-positive or Gram-negative) in a Universal bottle. The vessels were stirred at room temperature and viable counts performed at timed intervals using the 'Miles and Misra' method.[23] A bacterial control was included (either Gram-positive or Gram negative) for each experiment.

This methodology was used to test the biological activity of polymers halogenated at different times, temperatures and using different halogen concentrations. The bactericidal activity of chlorinated, brominated, and iodinated polymers were determined similarly.

Investigation of the mode of action of the *N*-halamine biocidal polymer

Halogen release as a mode of action of the *N*-halamine biocidal polymer

N-halamine biocidal polymer (**5**) (chlorinated) (0.5 g) was transferred to a section of sterile, rehydrated dialysis tubing (nominal molecular weight cut-off 12–14000 Da). The packet was immersed in a Universal bottle containing 10 mL of pre-incubated bacterial suspension grown for 17 h and the bacterial viability followed by counting at timed intervals. The experiment was performed on examples of both types of

TABLE II
The Halogen Load on the Polymers Prepared Under Different Conditions

	Chlorine (ppm)	Bromine (ppm)	Iodine (ppm)
Halogenated at different temperatures			
$< 5°C$	107 ± 0.7	169 ± 12	208 ± 20
Ambient temperature	120 ± 0.4	184 ± 4.3	222 ± 0
35°C	70 ± 0.7	176 ± 1.8	231 ± 2.9
45°C	64 ± 2.2	154 ± 2.9	101 ± 5.7
Halogenated at different times			
0.5 h	112 ± 1.7	154 ± 1.2	92 ± 0
1 h	122 ± 2.5	169 ± 11.4	208 ± 2.9
2 h	130 ± 20	186 ± 2.2	212 ± 0
4 h	141 ± 1.5	190 ± 0.7	218 ± 2.9
Halogenated at different concentrations of NaOX			
2.5% (w/w)	78 ± 0.7	110 ± 2.2	162 ± 0
5.0% (w/w)	118 ± 0.7	172 ± 2.9	208 ± 2.9
7.5% (w/w)	189 ± 0.7	198 ± 1.8	226 ± 5.8
10% (w/w)	210 ± 0.9	218 ± 3.2	231 ± 3.0

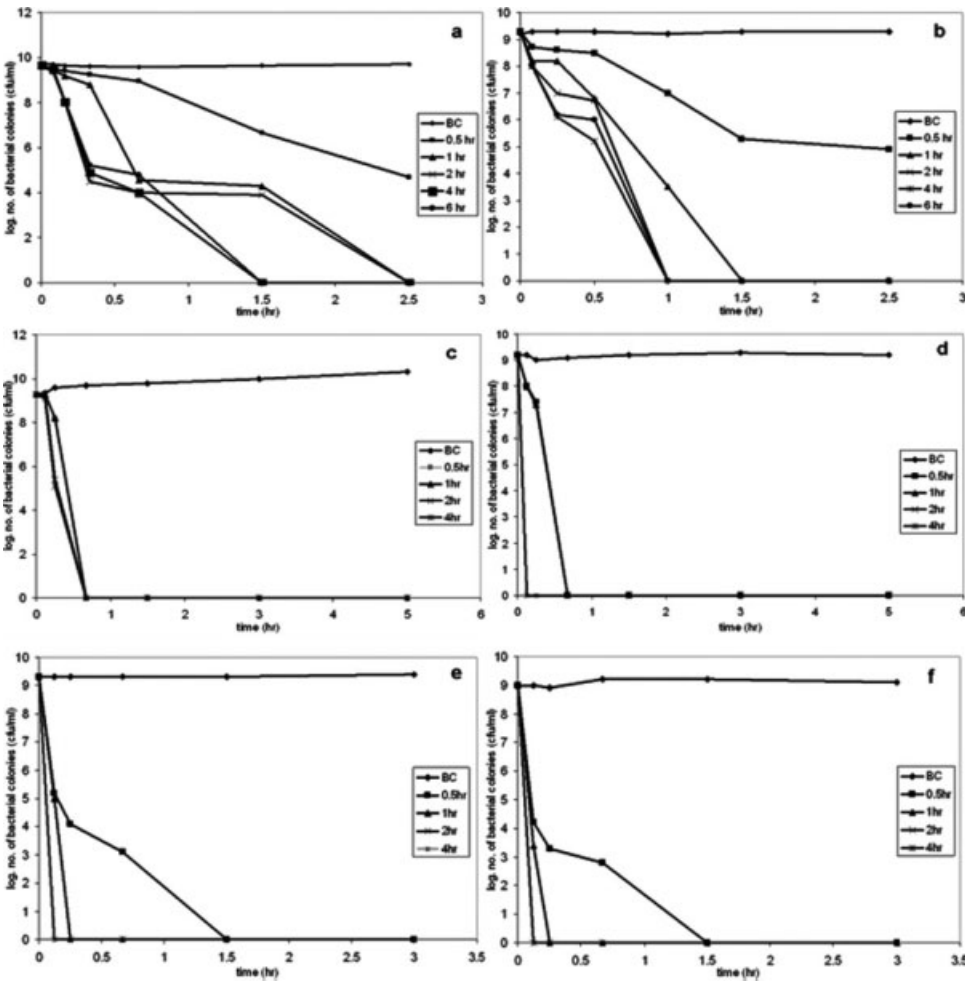


Figure 1 Viable counts of *E. coli* and *S. aureus* versus time after treatment with polymers halogenated for different time periods, chlorinated (a and b), brominated (c and d) and iodinated (e and f), where : BC = bacterial control-no treatment.

bacteria Gram-positive (*S. aureus*) and Gram negative (*E. coli*).

Investigation of contact as a mode of action of the *N*-halamine biocidal polymers

To investigate the "contact only effect", freeze-dried bacterial cells were challenged with polymer in the absence of liquid medium as follows:

Bacterial suspensions of *E. coli* or *S. aureus* were prepared by inoculating one bacterial colony into 50 mL nutrient broth in a 250-mL bottle and incubating for 17 h. The bacterial cells were collected by centrifugation (3500 rpm, 2383 g), washed twice with saline solution (0.9% w/w), frozen to −80°C overnight, and then freeze-dried under vacuum for 24 h to a dry powder.

Samples of polymer were compressed into small disks using KBr as a matrix to hold the polymer particles. Two disks containing the halogenated polymer **(5)** were prepared and a pre-weighed amount of the dried bacteria was placed between the two disks. Two further disks were prepared, containing the non-halogenated polymer **(4)** and a pre-weighed amount of bacteria, to be used as a control. A bacterial control was also included using the bacterial powder itself without polymer disks. All disks were left in contact with the bacteria for 3 h at which time some of the bacterial powder was removed aseptically from the disks, weighed, re-suspended in 10 mL of fresh nutrient broth and a viable count performed immediately.

Investigation of halogen transfer to bacterial medium as a possible mode of action

N-halamine chlorinated polymer **(5)** (0.3 g) was stirred in nutrient broth (15 mL) for 3 h. The polymer was removed and 10 mL of the medium divided into two parts (5 ml each); one inoculated with *E. coli* suspension (0.05 ml), pre-grown at 37°C for 17 h, and the other with *S. aureus* (0.05 ml, prepared under the same conditions). Growth was followed by counting at timed intervals and counts

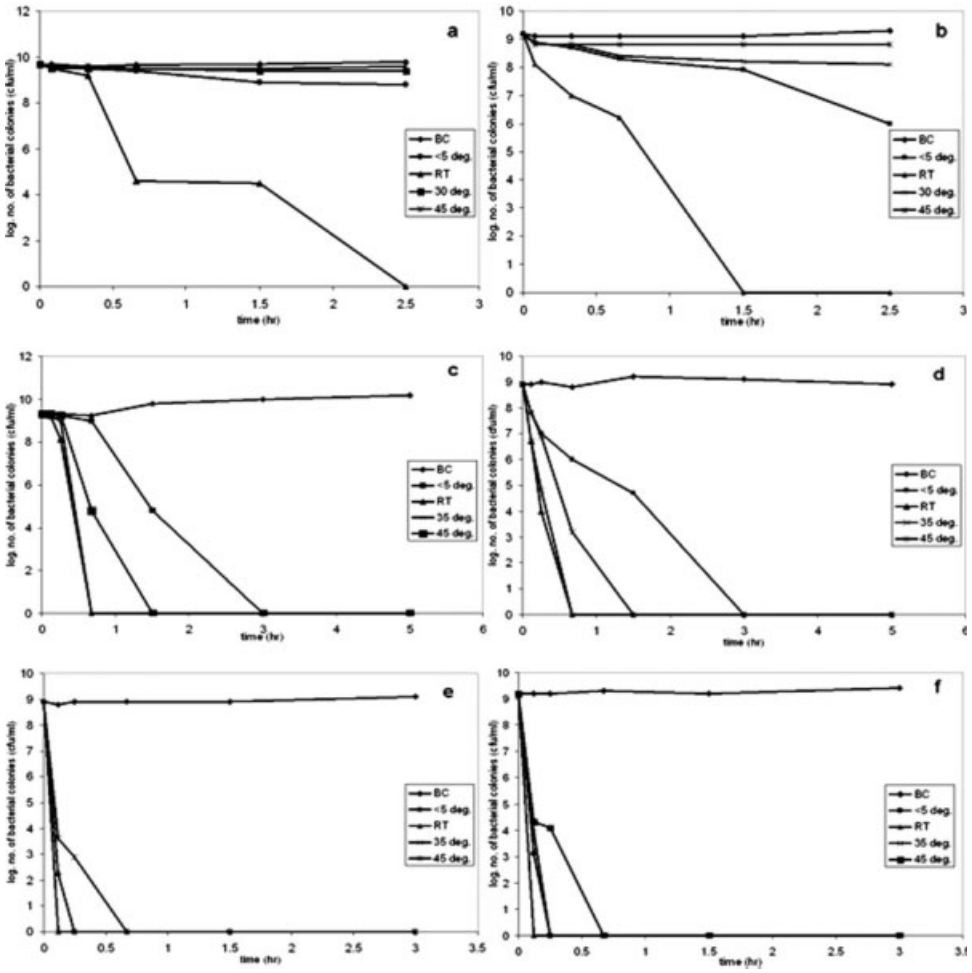


Figure 2 Log no. of *E. coli* and *S. aureus* colonies versus time after treatment with polymer halogenated at different temperatures: chlorinated (a and b), brominated (c and d) and iodinated (e and f), where : BC = bacterial control (no treatment); RT = ambient temperature.

compared to a bacterial control (for both *E. coli* and *S. aureus*) grown in fresh media.

RESULTS AND DISCUSSION

The biological activity of the *N*-halamine polymer halogenated under different conditions

Three conditions were investigated during halogenation; reaction temperature, sodium hypohalogenate concentration, and the time-period of halogenation. All other conditions were kept constant.

Halogenation time

For the chlorinated polymer **(5)**, as expected increasing the halogenation time up to 2 h increased the biological activity of the polymer against *E. coli;* while after 4 h the biological activity remained constant, Figure 1(a). The same effect was noticed for *S. aureus*, Figure 1(b). Similar behavior was seen in the case of bromination [Fig. 1(c,d)] and iodination [Fig. 1(e,f)]. For the brominated polymer, the biological activity increased with increasing bromination time to 2 h thereafter the biocidal activity remained constant for both *E. coli* and *S. aureus*, and the same pattern was recorded for the iodinated polymer. The quantity of the iodinated polymer used was reduced to 0.06 g (compared to 0.25 g for the chlorinated and brominated polymers) due to the higher biocidal power of this polymer. The conditions of bromination and iodination were kept the same as in the chlorination process (5% NaOX at lower than 5°C, while X = Br or I), Table I.

Halogenation temperature

For the chlorinated polymer, increasing the temperature from 0°C to room temperature increased the biological activity against *E. coli*, while above room temperature the biological activity decreased, this may be because above ambient temperature NaOCl becomes unstable which would decrease the halogen load on the polymer, Figure 2(a). The same behavior was observed with *S. aureus*, Figure 2(b).

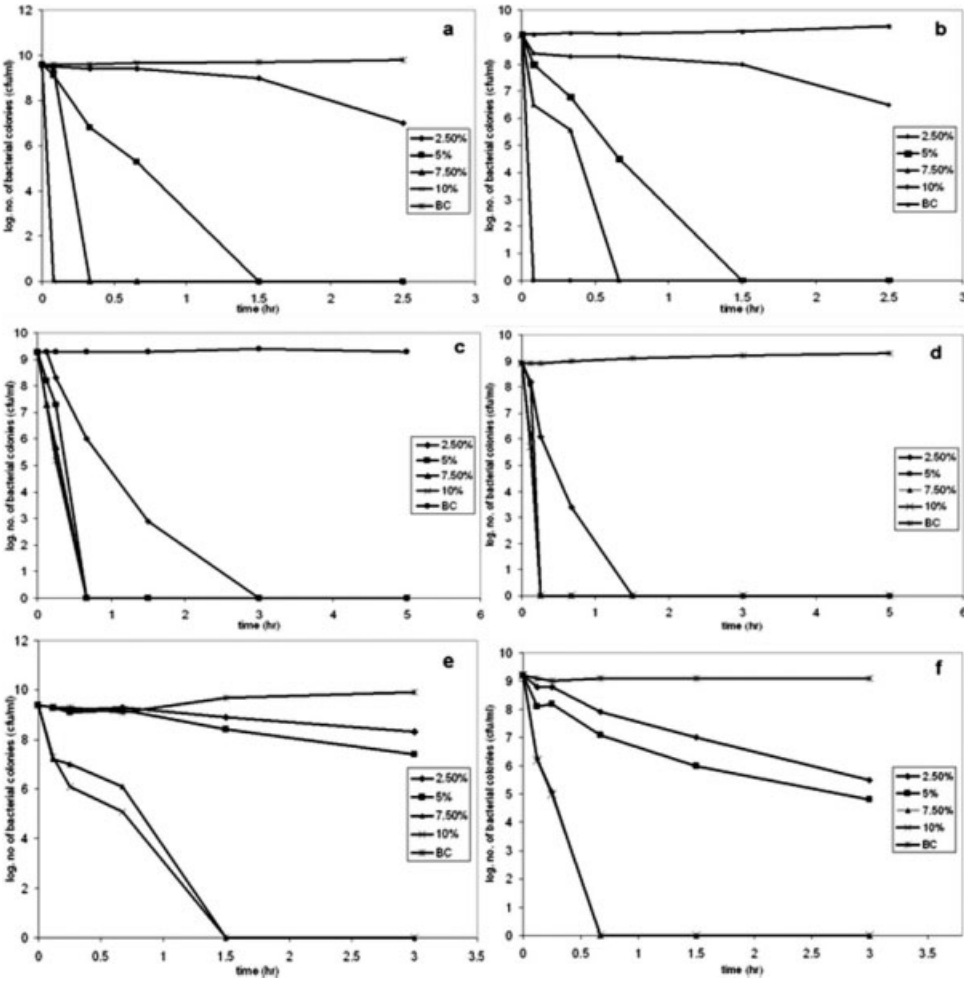


Figure 3 Log no. of *E. coli* and *S. aureus* colonies versus time for cells treated with polymers prepared with different concentrations of NaOCl (a and b), NaOBr (c and d) and NaOI (e and f), where BC = bacterial control-no treatment.

For the brominated polymer, the biological activity against *E. coli* increased with increasing halogenation temperature up to ambient temperature and stayed constant to 35°C; while above 35°C the biological activity began to decrease, Figure 2(c). The same behavior was seen for *S. aureus*, Figure 2(d). Similar results were recorded with the iodinated polymer for both *E. coli* and *S. aureus*, Figure 2(e,f). These results suggest that at elevated temperatures, the halogenation is ineffective, decreasing the biocidal activity, this is supported by the halogen-content of the corresponding polymers (Table II).

Halogen concentration

N-halamine polymer chlorinated with 10% NaOCl effects complete sterilization in just 5 min for *E. coli* and *S. aureus* [Fig. 3(a,b)] indicating, as expected, that increasing sodium hypochlorite concentration increased the biological activity of the polymer. Similar results were seen for both bromination [Fig. 3(c,d)] and iodination [Fig. 3(e,f)] and for both types of bacteria. The amount of iodinated polymer employed was reduced to 0.02 g polymer per 10 mL of the bacterial suspension due its powerful biocidal action.

From the results,

1. Two hours was sufficient to load the polymer with halogen; further increases in halogenation time did not shown any improvement in the biological power.
2. Increasing halogenation temperature from 0°C to room temperature increased the biological activity which then remained constant to 35°C for brominated and iodinated derivatives only. Therefore, halogenating the polymer at ambient temperature was considered to be most effective and practical procedure.
3. Within the range examined increasing the halogen concentration increased the biological action of the polymer. However, for bromination and iodination, maximum biocidal effect was achieved with a lower halogen concentration than for chlorination.

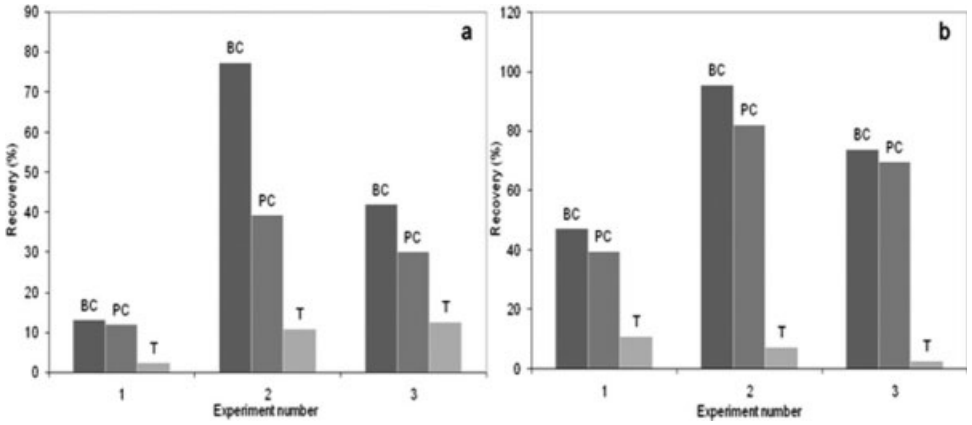


Figure 4 (a) and (b) Contact effect of the chlorinated polymer **(5)** on *E. coli* and *S. aureus* respectively in triplicates. BC = Bacterial control (cells without treatments); PC = polymer control (cells treated with non-halogenated polymer disks); T = tested polymer (cells treated with halogenated polymer disks).

4. Using NaOX under these conditions was more effective than using chlorine gas at 0°C. Moreover using NaOX reduces the costs and also the dangers associated with chlorine gas.

The biological results were supported by determining the chlorine load on the polymer using iodometric titration,[10,22] Table II. From these latter, it is clear that the amount of halogen loaded onto the polymer increased with time and sodium hypohalogenate concentration while increasing the temperature decreased the halogen load on the polymer, especially for the chlorinated polymers, Table II.

The mode of action of the *N*-halamine biocidal polymer

Over the last 20 years *N*-halamine biocidal polymers have been used in many applications.[1–22] The mode of action of this type of polymer has been investigated but until recently the mechanism whereby they exert their effect has remained unclear. Most of the work in this field has explained the bactericidal action by direct contact,[10,22] without free-halogen release while other workers explained it through halogen release into the media.[5]

However, our own work suggests it cannot be explained solely by means of one or the other, but by a combination of mechanisms operating simultaneously.

Our hypothesis for the bactericidal mode of action of these polymers is a combination of:

1. Direct contact between polymer and bacterial cell walls.
2. Release of halogen from polymer to the medium.
3. Transfer of halogen to medium constituents, which then affect bacterial growth and viability.

To investigate this hypothesis, we designed experiments which could provide evidence of these modes of action in isolation. To demonstrate the direct contact effect, bacterial suspension was freeze-dried and placed

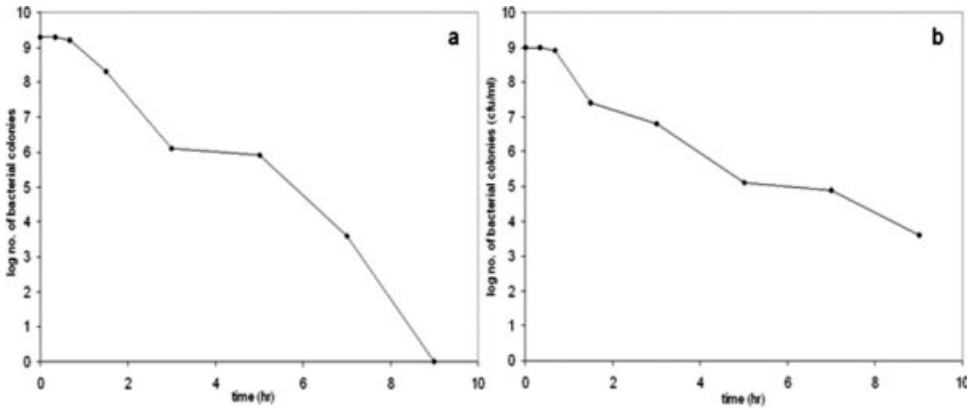


Figure 5 (a) and (b) The effect of the halogen release from the contained polymer on the bacterial viability of *E. coli* and *S. aureus* respectively.

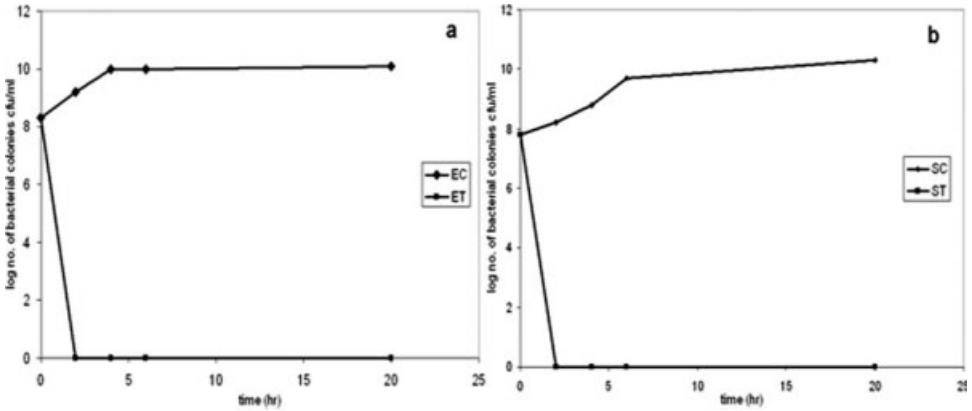


Figure 6 (a) and (b) Growth of *E. coli* and *S. aureus* respectively in a medium pre-treated with chlorinated polymer **(5)**, where EC and SC = *E. coli* and *S. aureus* bacterial controls, respectively. ET and ST = *E. coli* and *S. aureus* bacterial growth, respectively, in a pre-treated medium with the halogenated polymer.

between two disks of the halogenated polymer, **(5)** to measure the direct effect of the polymer on the bacteria without the presence of any liquid media necessary to mediate free-halogen release. Following the contact experiment the lyophilized bacterial cells, including controls, were recovered and viable counts performed. They were recorded as cfu/g of freeze-dried material and the results recorded as a % recovery – calculated as the following:

$$\text{Recovery} = \text{no. of bacteria after 3 hr contact time}/\text{original no. of bacteria}) \times 100$$

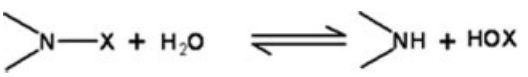
The original number of the bacterial cells was determined by re-suspending a weighed amount of the dried cells in nutrient broth and then performing a viable count, Figure 4(a,b).

The experiment was repeated three times, Figure 4. the extreme range of values seen are due to the variable distribution of bacterial cells through the solid residue after freeze-drying since the final powder will contain both the medium constituents as well as the bacterial cells. However, in all the three experiments the chlorinated polymer has reduced the bacterial viability of both *E. coli* and *S. aureus* which indicates that some biocidal action of the polymer is due to contact, Figure 4.

To provide evidence of bactericidal action without contact, the polymer was contained within a semi-porous membrane (dialysis tubing) permeable to soluble small species (nominal molecular weight cut of 12–14,000 Da), and placed within a bacterial culture. Since the polymeric species are totally insoluble and unable to permeate the membrane, any bactericidal effect would indicate release of active species, such as released-halogen.

For *E. coli*, Figure 5(a), it was noticed that the contained polymer effected a 9 log reduction in 9 h and for *S. aureus* a 5 log reduction in 9 h, Figure 5(b). The effect on *E. coli* being more pronounced than that on *S. aureus*.

Figure 5 clearly shows bactericidal action without direct contact of polymers and cells. When the polymer is placed in water, it should spontaneously release halogen according to the following release equlibrium equation:



Any species that can react with the free-halogen will upset this equilibrium causing further release. Thus the polymer would show controlled release of the dissolved halogen species; much more advantageous commercially than the use of free-hypochlorite, or similar species which have to be suitably dosed. This behavior has been referred to as 'bio-response'[5] but we prefer to consider it as a 'controlled-release' action. We have shown in our previous work that, in water, release of halogen was negligible but the amount of released halogen increased in the presence of bacterial cells.[2]

To provide evidence that the mode of action may be due to changing the nature of nutrients within the cultural medium, growth of *E. coli* and *S. aureus* was followed both in fresh broth and in nutrient broth pre-treated by exposure to chlorinated polymer **(5)** for 3 h. as described earlier, and subsequent removal of the polymer prior to inoculation of the medium.

Figure 6(a,b) show that the bacteria failed to grow in the medium treated with the halogenated polymer, even after the polymer has been removed, demonstrating that the polymer has effected some change on the nature of the nutrients in the media, possibly through halogen transfer. These results

support our previous work where we showed that the amount of released halogen increased when stirring halogenated polymer with nutrient broth compared to stirring in water.[2] One explanation may be changes occurring in the medium protein through halogen exchange between the polymer and the amide NH of the protein, transforming the latter and conferring biocidal properties. We are currently investigating this further.

The results indicate that the mode of action of these polymers is not by contact alone, or, release alone, but by a combination of factors; contact, halogen release, and transfer of halogen to medium components.

CONCLUSIONS

Halogenation parameters for an *N*-halamine polymer were investigated and the most practical and effective conditions described. Increasing the halogenation time (up to 2 h) as well as a halogen concentration of 10%, (10 mL per 1 g polymer) at ambient temperature increased the biological power of the polymer.

A combined mode of bactericidal action by *N*-halamine polymers, based on direct contact, halogen release and halogen transfer to medium components, has been described and is supported by the experimental evidence.

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